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14. ABSTRACT Ovarian cancer (OC) is the deadliest of all gynecological cancers, with five year survival rates of <45%. One critical feature of the disease is that two-thirds of the women diagnosed have advanced disease, and the five year survival rate of this group is <30%. This project outlines the development of a recombinant version of a member of a class of proteins known as disintegrins as an innovative imaging and diagnostic agent for ovarian cancer (OC). Vicrostatin (VN) is a recombinant protein based on the venom disintegrin contortrostatin (CN), which has shown impressive antitumor and antiangiogenic activities in models of human ovarian cancer. OC cells have been shown to display integrins $\alpha v \beta 5$ and $\alpha 5 \beta 1$, and the antitumor activity of CN, and demonstrated for VN, is based on the high affinity interaction between the disintegrin and these integrins. Thus far we have developed and shown that we have a robust and viable system for the production of VN and that the protein produced displays a high affinity for integrins displayed on ovarian cancer cells. In ongoing experiments we are evaluating the imaging potential for VN to be used for both evaluation of treatment and diagnosis of OC. The high affinity of VN for the integrins found on OC cells make for an excellent candidate for improvement of OC diagnosis and therapy.					
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Annual Progress Report

Introduction

The project entitled "Development of a Multifaceted Ovarian Cancer Imaging Agent" will cover the period of time from April 1 2007-March 31, 2011 (no cost extension, original end date 03/31/2010), this report covers the third annual period of the project April 1, 2009–March 31, 2011. The project focuses on the development of a production method for a recombinant disintegrin vicrostatin (VN), whose structure is based on the snake venom disintegrin contortrostatin (CN), and the use of the protein in imaging ovarian cancer. As a therapeutic agent the peptide is delivered via intra-peritoneal injection in a liposomal formulation. PET imaging radiotracers can be covalently attached to VN and used as an imaging and diagnostic agent in ovarian cancer (OC). In the previous reporting periods we utilized our expression system, described in first year's report, to produce VN for use as an imaging agent, further evaluated and quantitatively determined the integrin affinity of VN for a select group of integrins thought to be important in ovarian cancer and evaluated the circulatory half-life of both VN and liposomal encapsulated VN (LVN). Additionally, we have made progress on the development of VN as an OC imaging agent and have evaluated methods for radionuclide attachment to VN and made initial attempts to use the agent in PET imaging. We have shown that that the addition of PET tracer to VN does not alter its biological activity or ovarian cancer cell binding.

Summary of Progress on Specific Aims

During this year we were able to make significant progress toward successful completion of the goals and milestones of this project. Building on work from the previous years we were able to begin to develop PET imaging agents based on the disintegrins we have been studying. We were able to successfully label VN with ^{64}Cu and evaluate the retention of ovarian cancer cell binding affinity *in vitro*. Progress in these studies has lead to significant advances toward our final two milestones. Our Specific Aims and Milestones for this project are:

Specific Aim 1: Prepare VN, a recombinant disintegrin with proven *in vivo* antiangiogenic activity (**Milestone 1, completed Year 1**), and produce a liposomal formulation (LVN) with stability characteristics appropriate for clinical application (**Milestone 2, completed Year 1**).

Specific Aim 2: Demonstrate imaging potential and biological efficacy of a LVN formulation in a mouse model of ovarian cancer (**Milestone 3, in progress**).

Specific Aim 3: Evaluate the use of VN as a novel tumor imaging agent both for diagnostic use and for evaluation of tumor suppression following treatment (**Milestone 4, in progress**).

Body

Labeling VN for use as a PET Imaging Agent: For use as a PET imaging agent in ovarian cancer VN needs to be complexed with an appropriate PET radionuclide. In this instance the radionuclide utilized was ^{64}Cu , selected due to its relatively long (for a PET agent) half life, 12.7hrs, which makes it more suitable for larger peptide or protein based PET imaging, and more effective for conjugation to the macrocyclic chelator 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA), a heterobifunctional crosslinker that can be attached to Lys residues in proteins. To create a VN DOTA conjugate DOTA is first activated by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and N-hydroxysulfonosuccinimide (SNHS) at pH 5.5 for 30 min with a molar ratio of DOTA:EDC:SNHS = 10:5:4. The DOTA-succinimide reaction mixture (15 μmol , calculated on the basis of SNHS) was cooled to 4°C and added to VN (3:1 molar ratio) dissolved in 500 μL of water. The reaction mixture was adjusted to pH 8.5 with 0.1N of NaOH and was allowed to incubate overnight at 4°C. The DOTA-coupled VN was purified by semipreparative HPLC. The peak (detection at 220nm) containing the conjugate was collected, lyophilized, and dissolved in water (2 mg/mL) for use in radiolabeling reactions. For ^{64}Cu labeling of the VN:DOTA conjugate 20 μL of $^{64}\text{CuCl}_2$ (74 MBq in 0.1N HCl) was diluted in 400 μL of 0.1 mol/L sodium acetate buffer (pH 6.5) and added to the DOTA-VN solution (a 1 mg/mL solution of peptide was made and separated into aliquots; 80 μg of DOTA-VN per 37 MBq of ^{64}Cu were used for the labeling). The reaction mixture was incubated for 1 h at 40°C. ^{64}Cu -DOTA-VN was then purified by semipreparative HPLC, and the radioactive peak containing the desired product was collected. After removal of the solvent by rotary evaporation, the residue was reconstituted in phosphate-buffered saline for *in vivo* animal experiments. The labeling yield of ^{64}Cu -DOTA-VN is 74% based on the HPLC profile. For *in vitro* bioactivity studies cold CuCl_2 was substituted for the $^{64}\text{CuCl}_2$ all other procedures were carried out as with the radioactive material.

Retention of biological activity of Cu labeled disintegrin: To assess the biological activity of Cu-DOTA-VN we measured its ability to block processes critical to tumor survival and progression: adhesion, migration and invasion. We measured the inhibitory effect on different tumor cell lines, as well as endothelial cells. Inhibition of adhesion is evaluated through the ability of Cu-DOTA-VN to block cell attachment to a number of different extracellular matrix proteins. We observed a dose dependence in the inhibition of adhesion of OVCAR-5 and A2780 ovarian cancer cell lines as well as human umbilical vein endothelial cells (HUVEC), to both vitronectin and fibronectin, extra-cellular matrix (ECM) proteins that are ligands for integrins targeted by VN (data not shown). Cellular migration is also inhibited by VN in a dose dependent manner. To evaluate tumor and endothelial cell migration a phagokinetic tracking assay is employed. In this assay cells are plated on a collagen coated cover-slip with a an overlay of colloidal gold. As the cells move they displace or inject the colloidal gold leaving tracks on the surface of the cover-slip. Then, using dark-field microscopy the tracks can be visualized and photographed. Using image analysis software the area of the tracks in a photographed field can be determined and a "migration index" can be calculated as a percentage of the field lacking gold. Following treatment by increasing concentrations of VN the migration of both cancer and endothelial cells is significantly limited. Finally, the ability of cells to invade through the ECM was evaluated using modified Boyden chambers. These chambers contain a Matrigel coated porous membrane (pore size 8 μ m). A chemoattractant is placed in the lower chamber and untreated cells invade through the membrane toward the attractant. Both Cu-DOTA-VN and VN block the invasion of endothelial (HUVEC) and OVCAR-5 cells in a dose dependent manner with IC₅₀ at low nM concentrations. These results show that Cu-DOTA-VN has essentially identical activity to VN in inhibiting invasion of endothelial and ovarian cancer (OVCAR-5) cells. The results also convincingly demonstrate one of the important attributes of VN, that it inhibits endothelial cell as well as tumor cell invasion in the low nM range.

Binding of Cu labeled VN to integrins found on ovarian cancer cell surfaces: In the previous year's progress report we showed that recombinant VN binds with different affinities to a panel of human ovarian cancer cell lines dependent on the integrin display status of the individual cell line. In the present studies fluorescence polarization (FP) was used to determine binding kinetics to the identified subset of integrins. In this method, differing concentrations of functional integrin were incubated with a constant amount of Cu-DOTA-labeled FITC-VN, non radioactive Cu was used in these experiments but the process for labeling was carried out identically to that used with the PET tracer agent. As Cu-DOTA-FITC-VN is a small molecule it rapidly depolarizes the excitation light. Upon binding to the large integrin, the fluorescent tag on VN tumbles in solution at a slower rate resulting in increased levels of polarization. The measured FP value is a weighted average of FP values of the bound and free fluorescent VN and is therefore a direct measure of the fraction bound. Data generated in these experiments can be analyzed like standard radioligand binding, and kinetics of binding can be determined as with Scatchard analysis using a non-linear curve fit. From this set of experiments we determined the dissociation constants for FITC-VN and Cu-DOTA-FITC-VN with integrins α v β 3, α 5 β 1 and α v β 5. Cu-DOTA-FITC-VN and VN exhibit nearly identical affinities for α v β 3 (9.3nM and 7.4nM) α 5 β 1 (16.7nM and 15.2nM) and α v β 5 (43.6nM and 41.2nM). In the evaluation of the dissociation constants of Cu labeled VN for integrins α v β 3, α v β 5 and α 5 β 1 (integrins that are over expressed on ovarian cancer cell lines) we found that the addition of the crosslinker and Cu atom to the VN does not affect the affinities for the targeted receptors. The results of these studies support our hypothesis that the promiscuous nature of integrin binding by VN allows for broad targeting toward ovarian cancer, and that derivatization with Cu-DOTA will not adversely affect integrin binding by VN .

Preferential tumor binding by a recombinant disintegrin: In order to determine if there was preferential tumor binding by a recombinant disintegrin, VN the recombinant form of the venom derived disintegrin contortrostatin, was compared to a cyclic peptide, cyclo(-RGDfV-), similar to Cilengitide, which is currently in clinical trials for therapy of glioma. We evaluated VN as a PET imaging agent. This experiment was designed to show tumor specific binding of VN utilizing an existing bone metastasis model. In this case, androgen dependent prostate cancer cells were injected into the tibia of nude mice and were allowed to grow untreated for ~5 weeks until the tumors were 10-14mm in diameter. Animals were then injected with ⁶⁴Cu labeled or ⁶⁴Cu labeled peptide cyclo(-RGDfV), and imaged using a Concorde Systems micro-PET imaging system. As can be seen in **Figure 1**, injected VN localizes to the tumor with much higher specificity than the cyclic RGD peptide. This indicates that the recombinant disintegrin, VN, binds to the tumor and could serve as an effective imaging

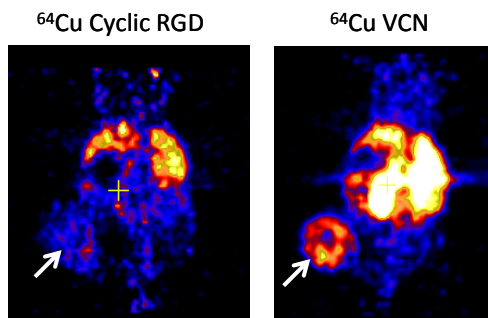


Figure 1 ^{64}Cu -VN binds to implanted bone tumor. Comparison of binding efficacy of ^{64}Cu labeled VN and a cyclic RGD peptide to an existing tumor implanted in the tibia of a nude mouse (white arrow points to tumor). As can be seen the VN binds with higher affinity than the RGD peptide to the existing tumor. This indicated that VN can potentially be used as both a therapeutic and or a PET imaging agent in cancer.

or therapeutic agent. While there is a high level of background in the reticuloendothelial system (RES), this can be overcome through two methods: first, masking to observe only the tumor under study, and second through serial images at longer time points than we attempted in this preliminary study. With masking, the tumor being treated can be identified and the region of interest evaluated would only be that of the tumor. In allowing more time for the labeled material to be removed from the body before imaging, the high affinity long-lived disintegrin bound to the integrin on the tumor cell would remain whereas the disintegrin associated with the RES would disappear.

Key Research Accomplishments

- Successfully labeled VN with both radioactive and non-radioactive copper
- Evaluated the retention of biological activity of VN following labeling
- Determined the binding affinities of copper labeled VN for soluble integrins important in ovarian cancer
- Performed comparative experiments of the potential for PET tumor imaging with copper labeled VN and a cyclic RGD peptide

Reportable Outcomes

1. Swenson, S., Minea, R. and Markland F.S. Development of an integrin targeted antiangiogenic agent. In: Tumor Angiogenesis: From Molecular Mechanisms to Targeted Therapy (F.S. Markland S. Swenson and R. Minea, Eds) Wiley-Blackwell, Weinheim Germany. Pg 181-206, 2010
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Conclusion

LVN is a novel liposomal formulation of a disintegrin engineered using standard recombinant techniques. The results from the Year I-III studies clearly show that LVN prepared by a commercially viable technique retains integrin binding and antiangiogenic activity equivalent to the laboratory prepared material. In the third year of this project we have been successful in determining the K_d of copper labeled VN for integrins important to OC. We have shown that integrins can be specifically labeled *in vivo*, and finally we have begun development of the methods necessary for use of VN as PET imaging agent.

References

None

Appendices

None